REMARKS

Formal Matters

Claims 104-113 were pending in this case prior to entry of the above amendment.

Claims 104 and 110 are newly amended. The amendments to the claims were made solely in the interest of expediting prosecution, and are not to be construed as acquiescence to any objection or rejection of any claim.

Claims 105 and 109 are hereby cancelled without prejudice or disclaimer.

No new claims are added.

Claim 104 has been amended to recite an additional step (c) that require normalizing the expression level of the target gene relative to the expression level of one or more reference genes in the tissue sample.

Support for normalizing the expression level of the target gene relative to the expression level of one or more reference genes in the tissue sample is found for example in paragraphs 160 and 184.

Claim 104 has been amended to recite an additional step (d) that requires comparing said normalized expression level of said target gene to data based on normalized expression of said target gene in cancer tissue samples obtained from patients of known clinical outcome.

Support for comparing said normalized expression level of said target gene to normalized expression of said target gene in cancer tissue samples obtained from patients of known clinical outcome is found in paragraph 0184 of the specification.

Claim 104 has been amended to recite an additional step (e) that requires determining a cancer prognosis or prediction for the human subject based on results obtained from step (d).

Support for determining a cancer prognosis or prediction for the human subject using the expression level of the target gene is found throughout the specification, including paragraphs 137, 184, 186 and 187 and Table 4.

Claim 110 has been amended to recite additional steps that require normalizing the expression level of the target gene relative to the expression level of one or more reference genes in the tissue sample, comparing said normalized expression level of said target gene to

normalized expression of said target gene in a cancerous tissue samples obtained from patients of known clinical outcome, and determining a cancer prognosis or prediction for the human subject based on results obtained from step (d). Support for these steps can be found throughout the specification, including paragraphs 160, 137, 184, 186-187 and Table 4.

Claims 104 and 110 are independent. The remaining claims depend, directly or indirectly, from independent claims 104 and 110.

Claim Objections

Claim 104 is objected to because it is missing a period at the end of the sentence. Claim 104 is currently amended and has a period at the end of the sentence

Claim Rejections under 35 USC, § 102 - Lipson

Claims 104, 110, 112 and 113 stand rejected under 35 USC, §102 as being anticipated by Lipson et al (PNAS 86: 9774-7 (1989).

Claim 104 has been amended to recite an additional step that requires normalizing the expression level of the target gene relative to the expression level of one or more reference genes in the tissue sample.

Lipson detects thymidine kinase (TK) hnRNA (intron) and TK mRNA (exon) and estimates and estimates the amount of each based densitometry of an autoradiograph and the number of cells used in a Southern blotting procedure. Lipson does not measure the expression level of a reference gene or use the expression level of a reference gene to normalize expression levels of a target gene.

Claim 104 has been amended to recite an additional step (d) that requires comparing said normalized expression level of said target gene to data based on normalized expression of said target gene in a cancerous tissue samples obtained from patients of known clinical outcome.

Lipson does not compare a normalized expression level of a target gene to data based on normalized expression of said target gene in a cancerous tissue samples obtained from patients of known clinical outcome.

Claim 104 has been amended to recite a further additional step that requires determining a prognosis or prediction for the human subject using the expression level of the target gene.

Lipson estimates the amount of TK hnRNA at different stages of the cell cycle after human fibroblasts have been cultured for 8 or more days ***in vitro*** in order to artificially synchronize their growth. Lipson's method does not employ a tissue sample that has been surgically removed from a human subject and does not provide a prognosis or prediction for a human subject. As such, Lipson's method is different to that being claimed, and this rejection should be withdrawn.

Withdrawal of this rejection is respectfully requested.

Claim Rejections under 35 USC, § 103 - Danenberg in view of Duvick, Clement, Lipson, Chang and Matsubara

The Examiner states that claims 104-109 and 111-113 are unpatentable over Danenberg in view of Duvick, Clement, Lipson, Chang and Matsubara. To the extent that this rejection applies to new or amended claims, Applicants respectfully traverse the rejection.

Claim 104 has been amended to recite an additional step that requires normalizing the expression level of the target gene relative to the expression level of one or more reference genes in the tissue sample.

Claim 104 has further been amended to recite an additional step that requires comparing said normalized expression level of said target gene to data based on normalized expression of the target gene in cancer tissue samples obtained from patients of known clinical outcome.

Claim 104 has also been amended to recite a further additional step that requires determining a prognosis or prediction for the human subject using the expression level of the target gene.

It is initially noted that claim 110 was not encompassed by this rejection. Claim 110 is re-written to be in independent form and, as such, it is believed that claim 110 is patentable. Allowance of claim 110 is respectfully requested.

In the previous Office Action, Examiner argues that the "only thing Danenberg does not teach is that the primers (and detection probe; see paragraph [0052]) were specific for intron sequences".

In order for primers and probe that are specific for intron sequences to be useful in the methods of the present invention, the introns must meet two criteria a) the intron must be detectable and b) the intron must be correlated with prognosis or prediction as the case may be.

Following his acknowledgement that Danenberg does not teach the primers (and detection probe) specific for intron sequences, Examiner quotes Duvick, "In this design, the spliced-out intron RNA would be detected at a level proportional to the transcription rate. Recent data from mammalian cells indicates that intron RNAs, after splicing, persist in the cell with reasonable half-lives, contrary to what was previously thought.

With regard to spliced-out introns persisting with reasonable half-lives, Duvick cites Clement, and in addition the Examiner has cited other publications including Lipson, Matsubara, and Chang. Examiner argues "the disclosures of Clement, Lipson, Matsubara, and Chang provide a reasonable expectation of success in quantitatively detecting intron sequences as a measure of gene expression". Applicants acknowledge the disclosures in Clement, Lipson, Matsubara, and Chang regarding detecting intron sequences. Applicants do not agree that detecting intron sequences as provided in the disclosures provides a reasonable expectation of success in quantitatively detecting intron sequences as a measure of gene expression

With regard to an intron being correlated with prognosis or prediction or being correlated with an exon which is correlated with prognosis or prediction, Duvick merely asserts (without presenting evidence) that in his experimental system, the spliced-out intron RNA would be ***detected*** at a level proportional to the transcription rate (emphasis added). In fact the spliced-out intron (as well as exons in the mRNA) would be ***synthesized*** at a level proportional to the transcription rate. To infer that the spliced-out intron RNA would be ***detected*** at a level proportional to the transcription rate, Duvick appears to assume that the degradation rate would be the same for the exon and for every spliced-out intron.

In fact, exons and introns are degraded independently. The intracellular RNA degradation machinery is largely exonucleolytic, allowing RNAs to escape decay simply by protecting their ends (Moore (2002) Cell 108, 431-434; Exhibit A). The 5' ends of most transcripts are protected by a methylated cap structure, which is added almost immediately after transcript initiation. The 3' end the mRNA is polyadenylated. Both the 5' cap and the 3' polyadenylation structure are further protected by binding proteins. Messenger RNAs (exons) then are degraded in the cytoplasm and their stability is controlled by caps, polyadenylation and other RNA stabilizing motifs (Janeau et al. (1999) RNA 5, 1119-29).

In the nucleus, the spliced-out intron are degraded by a complex pathway that is qualitatively different than for mRNA. This pathway involves formation and transient existence

of a circularized form of the introns ('lariats'). After the intron lariat is cleaved by an evolutionarily conserved debranching enzyme, the intron ends are not protected and the introns are degraded by nuclear exonuclease activity. Exonuclease activity in the nucleus is primarily 3'→5' exonuclease whereas exonuclease in the cytoplasm is primarily 5'→3' exonuclease (Bousquet-Antonelli, C et al (2000) Cell, 102, 765-775; Exhibit B). Intron degradation is fully described in Tollervey (2009) Cell 136, 763-776; abstract supplied as Exhibit C).

It should also be noted that, because exon sequences encode active protein, each mRNA species is expected to be regulated on an ongoing basis to reflect the particular biological requirements of cells. Introns do not have this functional restraint on their level maintained by cells. Although a few introns have regulatory roles or encode small nucleolar RNAs, the majority are thought to have no specific function (Moore (2002) Cell 108, 431-434). Consequently, their degradation would be expected to be regulated by different mechanisms than for mRNA. Therefore, the level of exon present and detected is not expected to be maintain a correlation with the level of exon present and detected.

One of skill would not combine the references in the manner proposed by the Examiner to provide the claimed method because they would have no reasonable expectation that introns would be degraded at the same rate as exons and would therefore be useful for prognosis or prediction as the case may be.

Furthermore, the method of Duvick is carried out in a experimental system, in which only the experimental variable, in this case the insertion site for the U-tag, is varied and in which other variables are controlled as closely as possible. For the reasons discussed above it is not reasonable to assume, even in a experimental system that an intron and an exon are degraded at the same rate and maintain correlation. Further, it is not warranted to extend Duvick's assumptions to patient tissues where many uncontrolled variables play a role in the separate degradation of intron sequences and exon sequences.

One of skill would not combine the references in the manner proposed by the Examiner to provide the claimed method because they would have no reasonable expectation that introns would be degraded at the same rate as exons in patient tissues and would therefore be useful for prognosis or prediction as the case may be.

In view of the above, the cited references do not support the rejection of claim 104 as amended.

Claims 106-108 and 110-113 depend, directly or indirectly, from claim 104, and thus inherit its limitations. For these reasons, the rejection of claims 106-113 is not supported by the cited references.

CONCLUSION

Applicants submit that all of the claims are in condition for allowance, which action is requested. If the Examiner finds that a telephone conference would expedite the prosecution of this application, please telephone James Keddie at (650) 833-7723.

The Commissioner is hereby authorized to charge any underpayment of fees associated with this communication, including any necessary fees for extensions of time, or credit any overpayment to Deposit Account No. 50-0815, order number GHDX-007.

Respectfully submitted,
BOZICEVIC, FIELD & FRANCIS LLP

Date: May 26, 2009

By: /James S. Keddie, Reg. No. 48,920/
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Exhibits: A Moore (2002)
B Bousquit-Antolelli (2000)
C Tollervey abstract

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Exhibit A

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Nuclear RNA Turnover

Minireview

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In mammalian cells, significantly more RNA is turned over in the nucleus than in the cytoplasm. However, only recently have we begun to understand the mechanisms and regulation of nuclear RNA decay.

Higher eukaryotes are remarkably wasteful in their metabolism of RNA. In mammals, only 50% of the nucleotides incorporated by RNA polymerase I (Pol I) end up in mature ribosomes. The remainder is trimmed away in the nucleolus by the pre-rRNA processing machinery. Pol II transcripts fair even worse, with less than 5% of their nascent phosphodiester bonds making it into cytoplasmic mRNA. Typically, nine out of ten nucleotides in human Pol II transcripts are removed as introns and recycled within the nucleus. The remaining exonic sequences are subject to additional quality control steps while still associated with the nucleus to eliminate mRNAs with incomplete open reading frames or other deformities (Figure 1). Although no definitive numbers exist that quantify how much Pol II-transcribed RNA is lost to such quality control, it has been estimated that only 50% of transcripts reaching the 3′ end of the dystrophin gene, the longest in the human genome, yield mature nuclear mRNAs (Jackson et al., 2000, and references therein). Pol III transcripts (e.g., 5S RNA and tRNAs) are also subject to trimming and splicing before exiting the nucleus. Thus, the bulk of RNA turnover in mammalian cells occurs in the nucleus, not the cytoplasm. However, in contrast to cytoplasmic mRNA decay, which has been studied extensively for the past decade as a key regulatory step in gene expression, nuclear RNA turnover is still largely unexplored territory. This minireview will focus on recent progress in this area.

The Nuclear RNA Degradation Machinery

Like intracellular protein degradation, intracellular RNA degradation must be closely regulated so as to prevent wholesale elimination of all transcripts. The intracellular RNA degradation machinery is largely exonucleolytic, allowing RNAs to escape decay simply by protecting their ends. The 5′ ends of Pol II transcripts are protected by a 7mGpppG cap structure, which is added almost immediately after transcript initiation, and then further protected via nuclear and cytoplasmic cap binding proteins. At the other end, the 3′ polyA tail is sequentially bound by nuclear and cytoplasmic polyA binding proteins. Other RNAs, such as rRNAs, snRNAs, snoRNAs, and tRNAs, carry alternate cap structures, tightly-bound proteins, and/or strong structural elements at their ends. An RNA can then be targeted for decay by deadenylation, decapping, displacement of bound proteins, disruption of its tertiary structure, or endonucleolytic cleavage at an internal site. Once an end is accessible, there exist both 3′ to 5′ and 5′ to 3′ exonucleases that can degrade it in either direction.

The nuclear RNA degradation machinery has been best characterized in budding yeast, where the predominant nuclear decay pathway is 3′ to 5′ (Bousquet-Antonelli et al., 2000). Most of this activity is provided by the exosome (van Hoof and Parker, 1999; Mitchell and Tollervey, 2000), also known affectionately as the "Death Star of RNA." Discovered in the Tollervey lab, the exosome is a complex of ten to eleven proteins, most of which have 3′ to 5′ exonuclease activity in vitro or are presumed exonucleases based on sequence homology. Accessory factors include RNA helicases, a putative GTPase, and proteins that target the exosome to particular RNA sequences. Initially characterized as the species responsible for 3′ end maturation of 5.8S rRNA, nuclear exosomes are also required for 3′ processing of snRNAs and snoRNAs and for the degradation of pre-rRNA spacer regions. All of the exosomal subunits are conserved in humans, where the complex is synonymous with the PM-Scl particle, a target of autoimmune antibodies (van Hoof and Parker, 1999; Mitchell and Tollervey, 2000, and references therein).

Exosomes also function in the cytoplasm, where they are required for 3′ to 5′ decay of deadenylated mRNAs (van Hoof and Parker, 1999). Cytoplasmic exosomes differ from their nuclear counterparts in that they lack one subunit, the nuclear protein Rrp6p. While the ten core subunits are all essential for growth, deletion of Rrp6p only causes temperature-sensitive lethality. Cells lacking Rrp6p activity accumulate stable nuclear RNAs with incompletely processed 3′ ends. Rrp6p has also been implicated in the turnover of nuclear pre-mRNAs and mRNAs, and the retention near the transcription site of mRNAs containing aberrant 3′ ends (Burkard and Butler, 2000; Hilleren et al., 2001; and see below).

There is also evidence for a 5′ to 3′ exonucleolytic decay pathway in the nucleus. *S. cerevisiae* contains two known 5′ to 3′ exonucleases, Rat1p and Xrn1p, both of which are conserved throughout eukaryotes. Rat1p is predominantly nuclear and is essential. It is known to be involved in pre-rRNA processing and snoRNA maturation, and may also function in mRNA maturation, as temperature-sensitive *rat1-1* cells rapidly accumulate nuclear polyA$^+$ RNA after shift to restrictive temperature. The Xrn1p enzyme provides the major decay pathway for deadenylated and nonsense-containing mRNAs in the cytoplasm, although it is not an essential protein. When their subcellular localizations are altered, both Rat1p and Xrn1p are functionally interchangeable, and even under normal conditions, Xrn1p appears to overlap Rat1p's functions in the nucleus (for references, see Geerlings et al., 2000; Xue et al., 2000). Although Rat1p and the exosome constitute the major nuclear decay machinery known to date, because our knowl-

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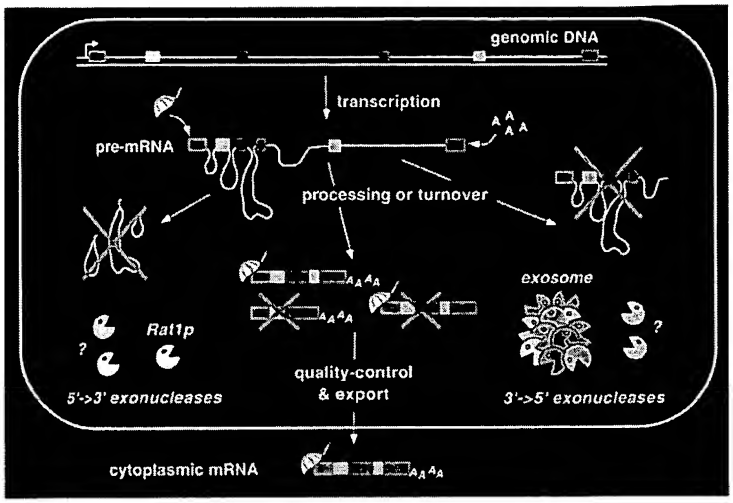

Figure 1. Nuclear Routes of Processing and Turnover for RNA Pol II Transcripts

Nuclear processing of Pol II transcripts includes capping, excision of introns, and addition of a polyA tail. The nuclear RNA degradation machinery consists largely of exonucleases, the best characterized of which are the exosome and Rat1p. In addition to degrading excised introns, nuclear exonucleases appear to assist in the elimination of inefficiently processed pre-mRNAs and malformed mRNAs.

edge is limited, there could well exist other important degradative enzymes that await characterization.

Nuclear Discard Pathways for Inefficiently and Improperly Processed Transcripts

In *S. cerevisiae*, mutations in pre-mRNA splicing factors can dramatically reduce spliced mRNA levels without concomitantly increasing the levels of unspliced pre-mRNAs. This implies the existence of an efficient pre-mRNA discard pathway. Recently, the Tollervey lab demonstrated that such a pathway does operate in *S. cerevisiae* (Bousquet-Antonelli et al., 2000). They found that when exosome mutations (*exo*$^-$) were combined with a strong splicing mutation (*prp2-1*), pre-mRNA levels rose 20- to 50-fold over the levels observed with the *prp2-1* mutation alone. Surprisingly, spliced mRNA levels also increased significantly in the *exo*$^-$ *prp2-1* cells over *exo*$^+$ *prp2-1* cells. These and other data suggest that the exosome normally degrades pre-mRNAs that are not efficiently processed, but when these pre-mRNAs are not degraded, they can be slowly spliced in the *prp2-1* background. Consistent with the notion that there is dynamic competition between splicing of nascent transcripts and their degradation by the exosome, disruption of exosome activity alone in an otherwise wild-type background increased the steady-state levels of both pre-mRNAs and spliced mRNAs. However, the same mutations had little effect on the steady-state level of an unspliced mRNA, suggesting that intronless mRNAs may not be efficient substrates for nuclear 3′ to 5′ decay in budding yeast.

In another recent study, the exosome was linked to a quality-control system for monitoring proper mRNA 3′-end formation. In *S. cerevisiae*, both hypo- and hyperadenylated mRNAs are retained at or near the site of transcription. Similarly, inefficiently processed transcripts in mammalian cells accumulate around the transcription site (Hilleren et al., 2001; and references therein). This suggests the existence of surveillance systems that inhibit transcription site release of RNAs that have not been properly processed. Remarkably, a recent study in *S. cerevisiae* revealed that mutation of three different exosome subunits, including the nuclear-specific subunit Rrp6, alleviated the transcription site accumulation of hypo- and hyperadenylated mRNAs (Hilleren et al., 2001). Thus, in addition to its exonucleolytic activities, another function of the exosome may be to retain at the site of transcription mRNAs that lack appropriate length polyA tails, or are otherwise malformed. Consistent with such a role, Rrp6 interacts genetically and physically with both the polyA polymerase and a nucleocytoplasmic shuttling protein, Npl3p, involved in mRNA export (Burkard and Butler, 2000). Further experiments will be required to determine whether the degradative and mRNA retention functions of the exosome are equally dependent on intact exonuclease active sites or are functionally separable. Also, it will be of great interest to investigate such quality-control pathways in mammalian cells, where the proliferation of alternative splicing and alternative polyadenylation are likely accompanied by significantly increased frequencies of processing errors.

Nonsense-Mediated mRNA Decay

A more extensively studied quality-control system than those described above is the nonsense-mediated mRNA decay (NMD) pathway (Maquat and Carmichael, 2001; Wilusz et al., 2001). This is a means for eliminating mRNAs with incomplete open reading frames, thereby preventing synthesis of potentially deleterious, truncated proteins. Such mRNAs can arise by genetic mutation (e.g., frameshift and nonsense mutations), through transcriptional and processing errors, or via the natural gene rearrangement process required for lymphocyte development. Studies in budding yeast are consistent with NMD being a purely cytoplasmic process. Supporting this idea are observations that NMD involves the normal translation apparatus, which upon recognition of a premature termination codon, triggers mRNA decapping without deadenylation and then 5′ to 3′ degradation via the cytoplasmic Xrn1 exonuclease pathway.

On the other hand, experiments in mammalian cells have clearly implicated the nucleus in NMD. Early experiments relying on biochemical separation of the nuclear and cytoplasmic compartments revealed that many mammalian mRNAs containing truncated open reading frames are found at lower levels than the corresponding

wild-type mRNAs in both nuclear and cytoplasmic fractions. A popular hypothesis to explain this phenomenon is that mammalian mRNAs are subject to reading frame recognition by cytoplasmic ribosomes coincident with nuclear export. In this way, a prematurely truncated open reading frame could be detected and the offending mRNA degraded before it is ever released from the nuclear pore complex. However, some recent studies have rekindled an old idea that there may actually be reading frame recognition and even translation within the nucleus itself. First, subcellular localization studies have shown that not only is Upf3p, a protein required for NMD, predominantly nuclear in mammalian cells, significant amounts of the cytoplasmic translation factors eIF4E and eIF4G are also found in the nucleus (for references, see Iborra et al., 2001; Ishigaki et al., 2001; Muhlemann et al., 2001). The nuclear fraction of the latter factors (~10% of total) is similar to the fraction of protein synthesis recently attributed to the nuclei of permeablized mammalian cells (Iborra et al., 2001). The nuclear association of newly made proteins observed by Iborra et al. is dependent on ongoing transcription, and RNA/protein colabeling techniques revealed that newly made nuclear proteins colocalize with newly made RNAs. If translation does occur in the nucleus, to what extent does it involve predominantly nuclear factors? A partial answer to this came from the Maquat lab, which showed that the first, or "pioneering," round of translation and this leads to NMD occurs while mammalian mRNAs are still bound by the nuclear cap binding complex and nuclear polyA binding protein (Ishigaki et al., 2001). Finally, the Moore and Wilkinson labs reported that disruption of mRNA reading frame can lead to accumulation of unspliced pre-mRNAs at or near the transcription site of the mutant allele (Muhlemann et al., 2001). This result opens the tantalizing possibility that, as in bacteria, reading frame recognition in mammalian cells might even occur cotranscriptionally. As of this writing, it remains to be determined whether the pre-mRNAs that accumulate upon disruption of reading frame represent molecules in line for mRNA synthesis and then NMD, or molecules that have somehow been targeted for destruction prior to splicing. Also, it is currently unknown what relationship, if any, exists between these transcription site accumulations and those observed in yeast for mRNAs with aberrant 3′ ends (Hilleren et al., 2001; see above).

If some or most NMD does occur inside the mammalian nucleus, it will be of great interest to determine which exonuclease pathway is involved. Does NMD of nuclear mRNAs involve deadenylation-independent decapping and then 5′ to 3′ decay, as does cytoplasmic NMD in *S. cerevisiae*? If so, what is the decapping enzyme and which 5′ to 3′ exonuclease ortholog (Rat1p, Xrn1p, or a novel enzyme) is involved? Or, given that the exosome is the more active exonuclease in the nucleus of budding yeast (Bousquet-Antonelli et al., 2000), perhaps mammalian NMD is a predominantly 3′ to 5′ degradation pathway. Recent work showing that the exosome is attracted to naturally short-lived mRNAs by proteins bound to instability elements (see below), might suggest a parallel mechanism for exosome attraction to mRNAs destined for NMD.

Intron Degradation

Even though introns represent up to 90% of the nucleotides incorporated by RNA Pol II in the nuclei of higher eukaryotes, until recently very little attention has been paid to the metabolism of these sequences once they have been spliced out. Although a few introns have regulatory roles or encode small nucleolar RNAs (snoRNAs), the majority are thought to have no specific function. After splicing, they must be disassembled from the spliceosome and degraded. Due to their lariat structure, a requisite step in intron degradation is cleavage of the 2′-5′ phosphodiester bond at the branch site. This reaction is carried out by an evolutionarily conserved debranching enzyme, deletion of which confers a severe growth defect in *Schizosaccharomyces pombe* (Nam et al., 1997). While lack of this enzyme is of little phenotypic consequence in *S. cerevisiae*, which has relatively few introns compared to *S. pombe* and higher eukaryotes, debranching-deficient *S. cerevisiae* cells do accumulate circular introns missing the lariat tail (Chapman and Boeke, 1991). This suggests that, like other nuclear RNAs, the main degradation pathway for debranched introns is an exonucleolytic one. However, which exonucleases are responsible is not yet known.

Recently, the Wilkinson lab showed that the half-lives of four mammalian introns after splicing were surprisingly long, ranging from 6 to 29 min. Such half-lives are comparable to naturally unstable mRNAs such as c-*myc* and c-*fos* (Clement et al., 2001; and references therein). Given that the longest intron in that study was 2.4 kb, and the half-lives of the introns they examined correlated loosely with length, it may now be of interest to determine whether longer introns have even longer half-lives and whether introns that are significantly more stable than the mean have any regulatory consequences for gene expression.

Nuclear Stabilization of Unstable mRNAs

Many mRNAs are naturally short-lived. This is an important control mechanism for temporally limiting the synthesis of the encoded proteins (e.g., cytokines and growth factors). Such mRNAs often contain instability elements in their 3′UTRs. One well-studied instability element is the ARE, or AU-rich element (Brennan and Steitz, 2001; Guhaniyogi and Brewer, 2001). Recently, it was shown that this element destabilizes an mRNA by attracting exosomes to it (Chen et al., 2001; Mukherjee et al., 2002). Exosomes are recruited through direct interaction with the ARE (Mukherjee et al., 2002), as well as through adaptor proteins that bridge the ARE and exosome (Chen et al., 2001). Given that exosomes purified from both nuclear and cytoplasmic compartments stimulate degradation of ARE-containing mRNAs (Chen et al., 2001), one wonders how ARE-containing mRNAs are ever able to run the gauntlet of exosomes in the nucleus and escape intact to the cytoplasm. The answer lies in the existence of repressor proteins that bind ARE sequences in the nucleus and prevent efficient exosome acquisition in that compartment. One such protein is HuR, a member of the ELAV family of RNA binding proteins. HuR is a predominantly nuclear shuttling protein, overexpression of which stabilizes ARE-containing mRNAs. In addition to fending off nuclear exosomes, HuR also helps shepherd ARE-containing mRNAs out of the nucleus via an alternate mRNA export pathway. Once in

the cytoplasm, HuR remains bound long enough for the ARE-containing mRNAs to be taken up into polysomes (Brennan and Steitz, 2001). Presumably, the eventual dissociation of HuR and its return to the nucleus then allows association of cytoplasmic exosomes with ARE-containing mRNAs, resulting in rapid decay. It is currently unknown whether HuR dissociation is a passive process dependent solely on its dissociation constant and lower concentration of HuR in the cytoplasm, or whether the translation machinery assists in HuR removal to actively limit the number of protein molecules translated from ARE-containing RNAs.

In summary, although our understanding of the machinery and regulation of RNA turnover in the nucleus is rapidly increasing, it is clear that many more revelations await. Among those revelations will undoubtedly be multiple surprises. Study of NMD in higher eukaryotes is already pushing our perceptions as to the extent to which RNA processing and translation are physically compartmentalized in eukaryotic cells. The elucidation of other nuclear decay pathways may well reveal a level of interplay between the RNA processing and decay machineries that we can only now just imagine.

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Exhibit B

Cell, Vol. 102, 7 5–775, September 15, 2000, Copyright ©2000 by Cell Press

Identification of a Regulated Pathway for Nuclear Pre-mRNA urnover

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Summary

We have identified a nuclear pathway that rapidly degrades unspliced pre-mRNAs in yeast. his involves 3′→5′ degradation by the exosome complex and 5′→3′ degradation by the exonuclease Rat1p. 3′→5′ degradation is normally the major pathway and is regulated in response to carbon source. Inhibition of pre-mRNA degradation resulted in increased levels of pre-mRNAs and spliced mRNAs. When splicing was inhibited by mutation of a splicing factor, inhibition of turnover resulted in 20- to 50-fold accumulation of pre-mRNAs, accompanied by increased mRNA production. Splicing of a reporter construct with a 3′ splice site mutation was also increased on inhibition of turnover, showing competition between degradation and splicing. We propose that nuclear pre-mRNA turnover represents a novel step in the regulation of gene expression.

Introduction

The yeast exosome is a protein complex that contains at least ten essential components (Rrp4p, Rrp40p, Rrp41p/Ski p, Rrp42p, Rrp43p, Rrp44p/Dis3p, Rrp45p, Rrp4 p, Mtr3p, Csl4p); all except Csl4p are known or predicted to be 3′→5′ exoribonucleases (Mitchell et al., 1997; Allmang et al., 1999a). The exosome and its human counterpart, the PM-Scl complex (Mitchell et al., 1997; Baker et al., 1998; Shiomi et al., 1998; Allmang et al., 1999a), are found both in the cytoplasm and the nucleus. The cytoplasmic exosome participates in mRNA turnover, whereas the nuclear exosome is involved in the processing and degradation of small nuclear RNAs (snRNAs), small nucleolar RNAs (snoRNAs), ribosomal RNAs (rRNAs), and pre-rRNA spacer fragments (Mitchell et al., 1997; Jacobs Anderson and Parker, 1998; Allmang et al., 1999b, 2000; van Hoof et al., 2000). An additional exosome component, Rrp p (PM-Scl 100), was specifically localized to the nucleus by immunofluorescence (Briggs et al., 1998; Allmang et al., 1999a; Burkard and Butler, 2000). Consistent with its nuclear localization, strains mutant for Rrp p do not exhibit defects in cytoplasmic mRNA turnover (van Hoof et al., 2000).

5′-end processing of snoRNAs and 5.8S rRNA as well as the degradation of rRNA spacer fragments involve the nuclear 5′→3′ exoribonuclease Rat1p (Amberg et al., 1992; Henry et al., 1994; Petfalski et al., 1998). Rat1p is essential for cell viability (Kenna et al., 1993; Johnson, 1997) and is functionally conserved from yeast to higher eukaryotes (Shobuike et al., 1995).

Control of mRNA stability is an important step in the regulation of gene expression. Two general cytoplasmic mRNA decay pathways have been characterized in yeast. In both, the initial step is shortening of the poly(A) tail to A_{10} or less (see Mitchell and Tollervey, 2000 and references therein). In the major pathway, this triggers removal of the 5′-cap structure by the decapping enzyme Dcp1p (Beelman et al., 199 ; LaGrandeur and Parker, 1998), exposing the body of the transcript to degradation by the cytoplasmic 5′→3′ exonuclease Xrn1p (Decker and Parker, 1993; Hsu and Stevens, 1993; Muhlrad et al., 1994, 1995), which is highly homologous to the nuclear exonuclease Rat1p (Johnson, 1997). An alternative 3′→5′ degradation pathway follows deadenylation and requires the activities of at least four exosome components, Rrp4p, Rrp41p/Ski p, Mtr3p, and Rrp44p (Jacobs Anderson and Parker, 1998; P. Mitchell and D. T., unpublished).

Aberrant mRNAs, including unspliced pre-mRNAs that escape to the cytoplasm, are rapidly degraded by pathways of mRNA surveillance or nonsense-mediated decay (NMD) (for reviews see Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Mitchell and Tollervey, 2000). In the yeast cytoplasmic NMD pathway, the context of the termination codon is sensed by a surveillance complex including Upf1p, Upf2p, and Upf3p. This can trigger rapid decapping and degradation by Xrn1p, independent of deadenylation (see Hilleren and Parker, 1999; Mitchell and Tollervey, 2000 and references therein). There are no reports of a role for the exosome or the nuclear exonuclease Rat1p in this pathway.

The existence of a nuclear turnover pathway for poly(A)$^+$ RNA in yeast has been proposed but never demonstrated (Burgess and Guthrie, 1993; de la Cruz et al., 1998; Burkard and Butler, 2000). Consistent with the existence of a such a pathway, mutated splicing reporter constructs are rapidly degraded in wild-type cells (Burgess and Guthrie, 1993), and many yeast splicing mutants show a strong reduction in mRNA levels without a corresponding accumulation of the unspliced precursor. Once intron-containing transcripts have been recognized and committed to splicing by commitment complex formation, they are restricted to the nucleus even when not spliced (Legrain and Rosbash, 1989; Rain and Legrain, 1997), indicating that they are degraded within the nucleus.

Using either a temperature-sensitive (TS) allele encoding the splicing factor Prp2p or reporter constructs with 3′ splice site mutations, we demonstrate the existence of a discard pathway for unspliced pre-mRNAs. In some cases, inhibition of pre-mRNA degradation increased the accumulation of spliced mRNA, suggesting that it competes with the splicing machinery. This discard pathway comprises two degradation mechanisms: a major 3′→5′ pathway requiring Rrp41p, Mtr3p, Rrp44p, and Rrp p, and most probably the entire exosome, and a minor 5′→3′ pathway involving Rat1p. The nuclear localization of this pathway is shown by the involvement

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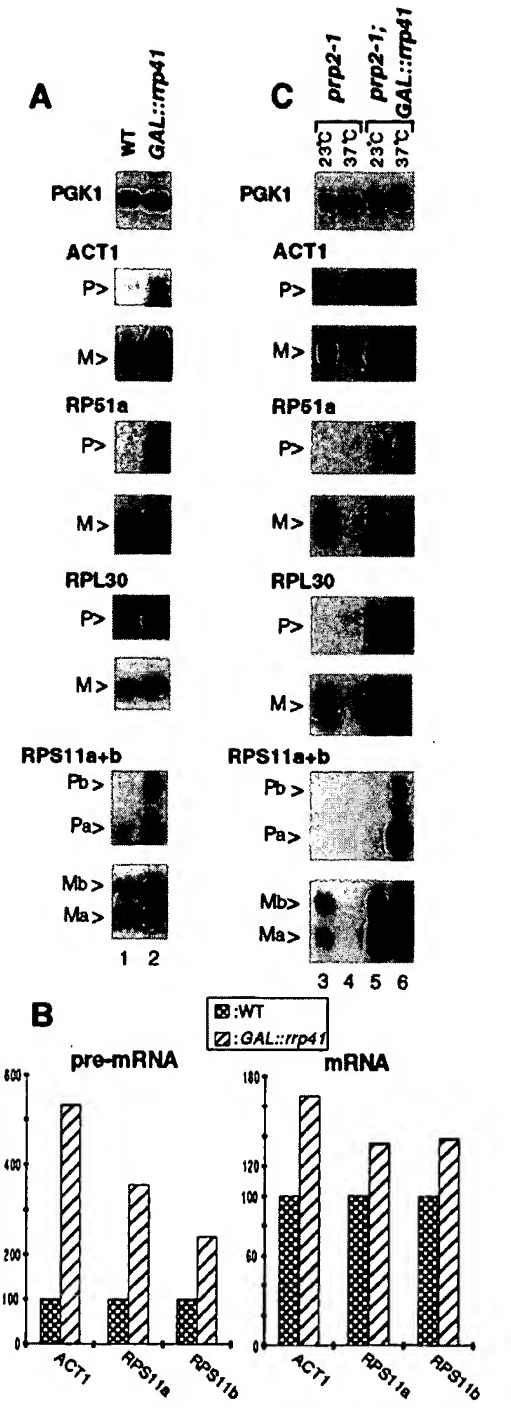

Figure 1. Depletion of Rrp41p Stabilizes pre-mRNAs

(A) Northern blot analysis of total RNA extracted from the *GAL::rrp41* (P118) and the isogenic wild-type (WT: YDL401) strains grown at 23°C in medium containing raffinose plus sucrose. The blot was hybridized successively with probes directed against intron containing pre-mRNAs, *ACT1* (400), *RP51a*: RP51a (419), *RPL30*: RPL30 (421), and *RPS11a* and *RPS11b*: RPS11a+b (420) and the intron less *PGK1* mRNA (418). The positions of the precursor (P) and mature (M) RNAs are indicated. For *ACT1* and *RPS11a* and *RPS11b*, panels showing the precursor form were exposed 2.5-fold longer than for the mRNA. For *RPL30*, a 5-fold longer exposure is shown. In all figures, the panels for each probe show lanes from a single Northern with the same exposure.

(B) PhosphorImager quantification of Northern hybridization data from (A). Checked bars correspond to the WT strain and striped bars to the *GAL::rrp41* strain. All pre-mRNA and mRNA levels have been normalized to the corresponding *PGK1* mRNA. The pre-mRNA and mRNA levels are expressed as a percentage of the corresponding pre-mRNA and mRNA WT levels, respectively.

(C) Northern blot analysis of total RNA extracted from strain *prp2 1* (YCBA20) and *prp2 1; GAL::rrp41* (YCBA30) grown in YP medium containing raffinose plus sucrose, either at 23°C (lanes 3 and 5) or for 3 hr at 37°C (lanes 4 and) to inactivate splicing. Hybridization was as for (A).

of the nuclear exonucleases Rat1p and Rrp p, the suppression of splicing defects and the lack of equivalent effects on inhibition of the cytoplasmic NMD pathway. Nuclear pre-mRNA turnover appears to compete with splicing in wild-type cells and to be subject to metabolic control, indicating that it normally functions as a regulated step in gene expression.

Results

Existence of a Nuclear Pre-mRNA Degradation Pathway Involving the Exosome Component Rrp41p

Strains depleted of Rrp41p/Ski p, or carrying the *ski6 100* TS mutation, are inhibited for 3′ processing of snRNAs, snoRNAs, and 5.8S rRNA, and for degradation of pre-rRNA spacer fragments (Mitchell et al., 1997; de la Cruz et al., 1998; Allmang et al., 1999a, , 1999b, 2000). The 3′ degradation pathway for cytoplasmic mRNAs is also inhibited by mutations in Rrp41p, but this does not lead to increased mRNA half-lives (Benard et al., 1998; Jacobs Anderson and Parker, 1998) since the cytoplasmic 5′ decay pathway is normally more active. RNA processing was initially analyzed under semipermissive conditions for Rrp41p expression. The *GAL::ProtA-rrp41* strain is able to grow in noninducing, nonrepressing raffinose, sucrose (RS) medium, but Western blot analysis on total protein showed that under such growth conditions, the level of the Protein A-Rrp41p fusion protein is strongly reduced (data not shown), leading to defects in 3′ processing of exosome substrates (Mitchell et al., 1997; Allmang et al., 1999a, 1999b, 2000).

Northern blot analysis was performed on total RNA extracted from the *GAL::ProtA-rrp41* strain and the isogenic wild-type (YDL401) grown in liquid RS medium (Figure 1A). Accumulation of all tested unspliced pre-mRNAs was observed in the Rrp41p-depleted strain (shown for *ACT1*, *RP51a*, *RPL30*, *RPS11a*, and *RPS11b* in Figure 1A). Moreover, this was accompanied by modest increases in the spliced mRNAs as compared to the nonspliced *PGK1* mRNA. Figure 1B shows PhosphorImager quantification of the data in Figure 1A. Quantitation showed that on depletion of ProtA-Rrp41p the *ACT1* pre-mRNA accumulated from 5- to 8-fold in different experiments, while *RPS11a* pre-mRNA accumulated 3- to 4-fold and *RPS11b* accumulated 1.8- to 2. -fold. The corresponding mRNAs were also mildly accumulated (1.3- to 1.7-fold). In Figure 1A, RNA loading is standardized to total RNA. In Figure 1B, the signals have been standardized to the nonspliced *PGK1* mRNA in the same samples. Very similar results were obtained in each

Exhibit B

case. The increased mRNA levels make it likely that pre-mRNA accumulation is nuclear and unlikely that this is due to a splicing defect. The large magnitude of the increase in pre-mRNA levels, and discrepancy between pre-mRNA and mRNA, make it most unlikely that this is due to increased transcription.

Much more dramatic pre-mRNA accumulation was observed when partial depletion of Rrp41p was combined with a *prp2 1* TS mutation (Figure 1C). Prp2p is a member of the DEAH box family of putative ATP-dependent RNA helicases that is required prior to the first transesterification reaction and is released from the spliceosome following ATP hydrolysis (Plumpton et al., 1994). Prp2p is not required for spliceosome assembly and the spliceosome remains intact and associated with the pre-mRNA in *prp2 1* strains (King and Beggs, 1990; Kim and Lin, 199). Pre-mRNA and mRNA accumulation was compared in *prp2 1* and *prp2 1; GAL::rrp41* strains at 23°C and 3 hr after transfer to 37°C to inactivate splicing. In the *prp2 1* strain at 37°C the levels of all tested spliced mRNAs (*ACT1*, *RP51a*, *RPL30*, *RPS9a*, *RPS9b*, *CYH2*, *RPS11a*, *RPS11b*, *SEC14*, and *SAR1*) were strongly reduced, whereas the level of the non-spliced *PGK1* mRNA was unaffected (Figure 1, lane 4; Figure 2, lane ; and data not shown). Little accumulation of the unspliced pre-mRNAs was seen, consistent with their rapid degradation. When splicing was inhibited in the Rrp41p-depleted strain, all tested pre-mRNAs were strongly accumulated, approximately 20- to 50-fold (tested for *ACT1*, *RP51a*, *RPL30*, *RPS9a*, *RPS9b*, *CYH2*, *RPS11a*, *RPS11b*, *SEC14*, and *SAR1*) compared to the *prp2 1* single mutant (Figure 1C, lanes 4 and and data not shown). For *RPS11a* (Figure 1C, lane) and *CYH2* (Figure 3, lane 4), this was accompanied by a restoration of the level of the mature mRNA to close to wild-type levels; some reaccumulation of the *RPS9a* and *RPS9b* mRNAs was also seen (Figure 3). Figure 1C shows growth in semipermissive RS medium; Figure 3 shows the effects of transfer from permissive, galactose medium, to nonpermissive, glucose medium.

RPL30 pre-mRNA accumulated strongly in the *GAL::rrp41* strain and in the *prp2 1; GAL::rrp41* strain at permissive temperature (Figure 1C, lane 5; Figure 3, lane 3). *RPL30* encodes ribosomal protein Rpl30p and is subject to feedback inhibition of splicing by free Rpl30p (Eng and Warner, 1991; Vilardell and Warner, 1994). The pre-mRNA is therefore probably normally inefficiently spliced, with nuclear degradation of the pre-mRNA.

To confirm that the unspliced pre-mRNAs were not degraded in the cytoplasm, we examined strains defective in the cytoplasmic NMD degradation pathway. In the *upf1-Δ* strain (Figure 2, lanes 3 and 4), accumulation of pre-*CYH2*, a well-characterized NMD substrate, was seen. Accumulation of pre-*RPL30* was seen at 23°C, but not at 37°C, and no clear accumulation was seen for other pre-mRNAs tested (*RPS11a*, *RPS11b*, *ACT1*, *BEL1*). In the *prp2 1*; *upf1-Δ* strain at 37°C (Figure 2, lane 8), no clear accumulation of any unspliced pre-mRNA was seen compared to the *prp2 1* single mutant (Figure 2, lane). Indeed, pre-*CYH2* levels were lower in the *prp2 1*; *upf1-Δ* strain than in the *upf1-Δ* single mutant; we speculate that the assembly of defective spliceosomes in the *prp2 1* strain may efficiently target the pre-mRNA for nuclear degradation.

We conclude that unspliced pre-mRNAs are rapidly degraded in the yeast nucleus and that this requires the activity of the 3′→5′ exonuclease Rrp41p. This pathway degrades a fraction of the unspliced pre-mRNAs in wild-type cells and is very active when splicing is inhibited by the *prp2 1* mutation. The degree of accumulation of different pre-mRNAs was not identical, suggesting that nuclear pre-mRNAs have different rates of turnover in wild-type cells. This is perhaps analogous to the different turnover rates seen for cytoplasmic mRNAs.

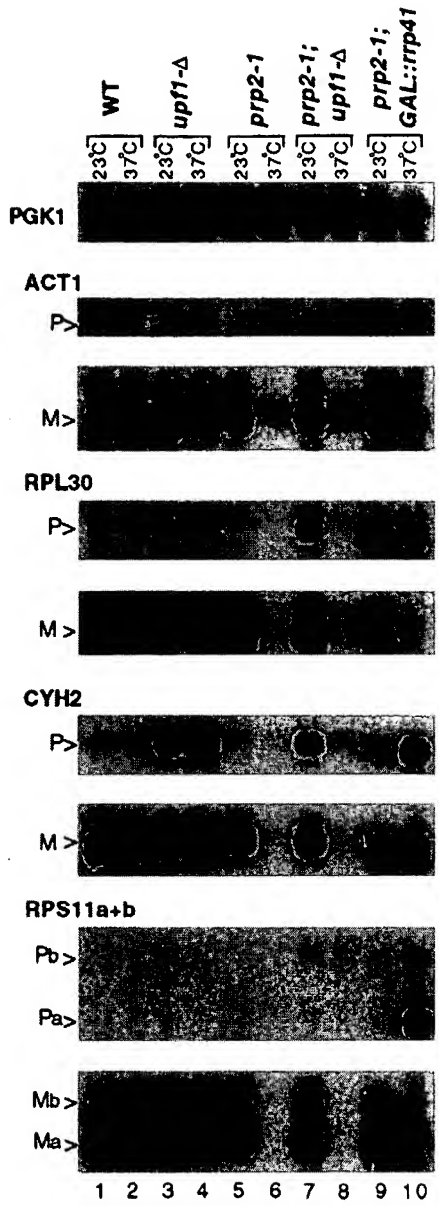

Figure 2. Inactivation of Upf1p Does Not Stabilize Pre-mRNAs in a Splicing-Deficient Strain

Northern analysis of total RNA extracted from WT (D271) (lanes 1 and 2), *upf1-Δ* (YCBA73) (lanes 3 and 4), *prp2 1* (YCBA20) (lanes 5 and), *prp2 1; upf1-Δ* (YCBA75) (lanes 7 and 8), and *prp2 1; GAL::rrp41* (YCBA30) (lanes 9 and 10) grown in YPD at permissive temperature (23°C) or for 3 hr at nonpermissive temperature (37°C). The same blot was hybridized with multiple probes; oligos are as described for Figure 1.

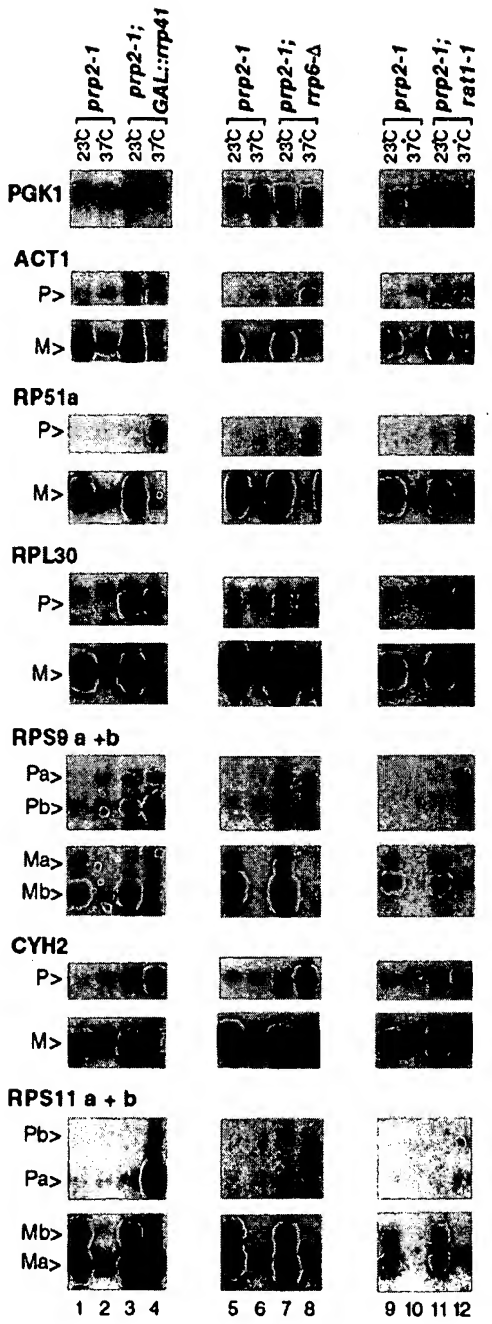

Figure 3. Inactivation of Rrp41p, Rrp p, or Rat1p Stabilizes Pre-mRNAs in a Splicing-Deficient Strain

Northern analysis of total RNA extracted from the *prp2 1* (YCBA20), *prp2 1; GAL::rrp41* (YCBA30), *prp2 1; rrp6-Δ* (YCBA27) and *prp2 1; rat1 1* (YCBA57) strains. Hybridization probes used were as for Figure 1, panels for *RPS9a* and *RPS9b* (422) and *CYH2* (405) have been included. For lanes 1 to 4, strains were pregrown in galactose medium at 23°C, transferred to glucose medium at 23°C for 20 hr (lanes 1 and 3), and then shifted to 37°C for 3 hr (lanes 2 and 4). For lanes 5 to 12, strains were pregrown in glucose medium at 23°C (lanes 5, 7, 9, and 11) and then shifted to 37°C for 3 hr (lanes , 8, 10, and 12).

he Nuclear Decay Pathway Requires the Activities of the Nuclear Exosome Complex and Rat1p

Protein A-Rrp41p is predominately present as a component of the exosome complex rather than as a free protein (P. Mitchell and D. Tollervey, unpublished), suggesting that the nuclear exosome complex is responsible for pre-mRNA degradation. To confirm this, we deleted the gene encoding Rrp p in a *prp2 1* strain (see Experimental Procedures). Rrp p is associated with only the nuclear form of the exosome complex and is nonessential, although cells lacking Rrp p are impaired in growth and TS-lethal (Briggs et al., 1998). Compared to the single *prp2 1* mutant strain, in the *prp2 1; rrp6-Δ* strain ten intron-containing pre-mRNAs tested were accumulated approximately 5- to 11-fold (shown for *ACT1*, *RP51a*, *RPL30*, *RPS9a*, *RPS9b*, *CYH2*, *RPS11a*, and *RPS11b* in Figure 3, lanes and 8; plus *SEC14* and *SAR1* data not shown). Clear reaccumulation of the mature *CYH2* mRNA was also seen. Pre-mRNA accumulation was stronger on depletion Rrp41p than in the absence of Rrp p; greater inhibition of the activity of the nuclear exosome in pre-snRNA and pre-snoRNA processing was also seen on Rrp41p depletion (Allmang et al., 1999b).

Two homologous 5′→3′ exonucleases, Xrn1p and Rat1p, participate in RNA processing and degradation. Rat1p is predominately nuclear (Johnson, 1997) and participates in 5′ processing of snoRNAs and rRNA, as well as the degradation of pre-rRNA and pre-snRNA spacer fragments (Henry et al., 1994; Petfalski et al., 1998). The *prp2 1* mutation was therefore combined with the TS *rat1 1* allele (Amberg et al., 1992). Weaker stabilization (2.5-fold) of several pre-mRNAs was seen, together with some increase in the levels of the mature *RPL30* and *CYH2* mRNAs (Figure 3, lanes 10 and 12).

We conclude that pre-mRNA degradation involves the nuclear exosome complex and the nuclear 5′→3′ exonuclease Rat1p.

Competition between Pre-mRNA Degradation and Maturation

For several genes tested, inhibition of pre-mRNA degradation was accompanied by increased mRNA levels, suggesting a competition between the splicing and degradative pathways. To confirm this, we analyzed the splicing of reporter constructs with mutations at the 3′ splice site. Plasmid pJU83 carries an *ACT1-CUP1* reporter construct (Figure 4A), while plasmids pJU97 and pJU98 have single nucleotide substitutions at the 3′ splice site that inhibit the second catalytic step of splicing (Lesser and Guthrie, 1993). These were transformed into strains carrying TS mutations in the exosome components *mtr3 1* or *rrp44 1*, the *rat1 1* strain and the wild-type control strain, and analyzed by primer extension (Figure 4B).

No significant difference in accumulation of mRNA synthesized from the wild-type *ACT1-CUP1* reporter construct was seen in the different genetic backgrounds (Figure 4B, lanes 1 to 8). As for endogenous genes, some pre-mRNA accumulation was seen in the *rrp44 1* and *rat1 1* strains. No signal was seen for the nontransformed wild-type strain (data not shown).

In the wild-type strain, splicing of the *ACT1-CUP1* construct was strongly inhibited by the 3′ splice site mutations, although not totally blocked. For both constructs, increased mRNA production was seen in the

Exhibit B

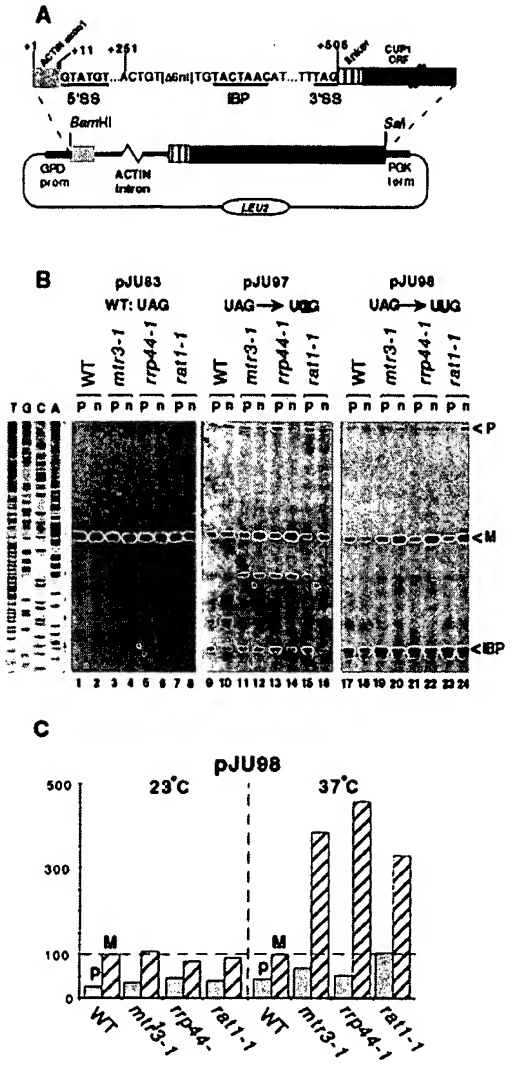

Figure 4. Splicing of Mutant *ACT1-CUP1* Reporter Constructs Is Increased by Inhibition of Nuclear Pre-mRNA Degradation

(A) Structure of the *ACT1-CUP1* construct. Exon 1 and the intron of the *ACT1* gene are fused to the *CUP1* ORF (Lesser and Guthrie, 1993). A nt deletion (Δ nt) in the actin intron removed a cryptic branchpoint (Vijayraghavan et al., 198). Sequences of the 5′ splice site (5′SS), the intron branch point (IBP) and the 3′ splice site (3′SS) are in bold and underlined. The construct is under control of the *GPD* promoter (GPD prom) on a *LEU2*-2μ plasmid.

(B) Primer extension analysis. A wild-type (pJU83) construct and two constructs with 3′SS mutations (pJU97 and pJU98) were analyzed in wild-type (WT, D271), *mtr3 1* (YCBA55), *rrp44 1* (P213), and *rat1 1* (D1 2) strains. RNA was extracted following growth at the permissive temperature (p) of 23°C or 3 hr after transfer to the nonpermissive temperature (n) of 37°C. Positions of reverse transcriptase stops corresponding to the 5′ end of the pre-mRNA (P), the 5′ end of the mRNA (M), and the intron branch point (IBP) are indicated. Primer extension was performed with oligo 425, complementary to +34 to +11 of the *CUP1* ORF.

(C) PhosphorImager quantification of pre-mRNA and mRNA levels of pJU98 at 23°C and 37°C from panel (B). The levels of pre-mRNA (gray gradient bars) and mRNA (striped bars) are shown relative to the corresponding RNAs in the wild-type strain, arbitrarily set at 100.

mtr3 1, *rrp44 1*, and *rat1 1* strains at 37°C (Figure 4B). Quantification of the data for pJU98 (Figure 4C) showed that, compared to the wild-type strain, there is 3.8-fold more mRNA in the *mtr3 1* strain, 4.5-fold more mRNA in the *rrp44 1* strain and 3.3-fold more mRNA in the *rat1 1* strain grown at 37°C. Very little difference was seen at 23°C.

For both mutant constructs, a primer extension stop at the intron branch point (IBP) was detected, due to accumulation of the intron lariat-exon 2 splicing intermediate (Figure 4B). Quantitation showed that the level of the IBP was increased 2- to 3-fold in the exonuclease mutants compared to the wild-type. The level of the IBP was reduced 1.3- to 1.7-fold in the mutant strains at 37°C compared to 23°C; however, a 1.2-fold decrease was seen in the wild-type and the significance of this is unclear.

We conclude that the nuclear turnover pathway also degrades incompletely spliced intermediates. As for the full-length pre-mRNA, splicing of these intermediates is in competition with the turnover pathway.

Reporter constructs with mutations at the 5′ splice site and intron branch point region were also analyzed (see Experimental Procedures for mutations used). In contrast to the 3′ splice site mutations, little or no suppression of splicing of these pre-mRNAs was observed in the *rrp44 1* strain, and pre-mRNA accumulation was similar to the wild-type construct (data not shown). Mutation of reporter constructs in the 5′ splice site and branch point region are reported to induce a defect in splicing commitment and allow export of the pre-mRNA to the cytoplasm (Legrain and Rosbash, 1989; Rain and Legrain, 1997). We conclude that mutations in Rat1p or in exosome components lead to stabilization of unspliced pre-mRNAs only if they are restricted to the nucleus.

Relative Contributions of 5′ and 3′ Degradation

To determine the relative contribution of the 5′ and 3′ pathways to pre-mRNA turnover, we analyzed the intermediates generated by blocking progression of the exonucleases.

Many snoRNAs are encoded within introns of pre-mRNAs and are released by processing (reviewed in Venema and Tollervey, 1999). The U24 snoRNA is processed from the intron of the *BEL1* gene after its release by splicing and debranching of the intron lariat (Petfalski et al., 1998). The mature snoRNA is generated by exonuclease digestion from both ends of the intron, catalyzed by Rat1p and the nuclear exosome (Petfalski et al., 1998; Allmang et al., 1999b), with final 3′ trimming by the Rrp p component of the exosome (Allmang et al., 1999b; van Hoof et al., 2000). Since nuclear pre-mRNA degradation and snoRNA maturation involve the same exonucleases, it appeared probable that the presence of an intronic snoRNA and associated proteins would stall pre-mRNA degradation.

The degradation of *BEL1* was analyzed in *prp2 1* strains depleted of Rrp41p (*prp2 1*; *GAL::rrp41*), lacking Rrp p (*prp2 1*; *rrp6*-Δ), or with the TS Rat1p mutation (*prp2 1*; *rat1 1*). Probes used are numbered 1–5 in Figure 5A. In the *prp2 1* strain at nonpermissive temperature (Figure 5B lanes 2, , and 10) four RNA species were detected—the full-length pre-mRNA (P), two 3′ truncated intermediates (A and A′), and the 5′ truncated intermediate (B). Primer extension analysis (not shown)

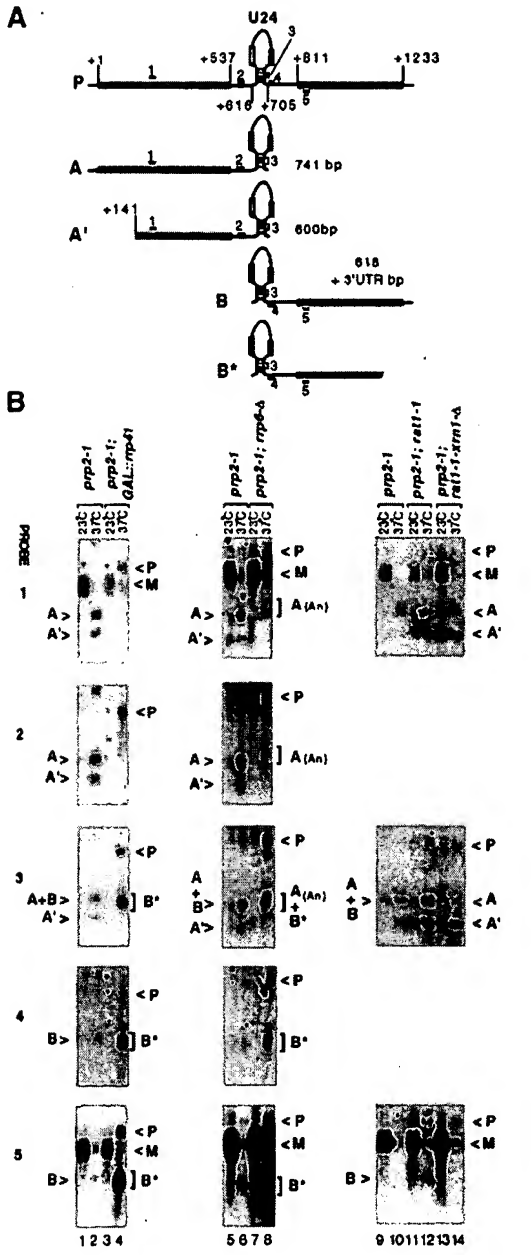

Figure 5. Degradation of a Pre-mRNA Containing an Intronic snoRNA

(A) Schematic representation of the pre-mRNA degradation intermediates observed for *BEL1* (the host gene for U24). P: full-length pre-mRNA; A: product of 3′→5′ degradation that extends from the 5′ end of the transcript to the 3′ end of U24; A′: intermediate A truncated at position +141. B: product of 5′→3′ degradation, that extends from the 5′ end of U24 to the 3′ end of the transcript. B*: intermediate B partially digested from its 3′ end. The locations of the hybridization probes are indicated. 1 (U24–5′ex: 784), 2 (U24–5′int: 785), 3 (U24: 214), 4 (U24–3′int: 213), and 5 (U24–3′ex: 423).

(B) Northern blot analysis of RNA extracted from strains *prp2 1* (YCBA20) (lanes 1, 2, 5, , 9, and 10), *prp2 1; GAL::rrp41* (YCBA30) (lanes 3 and 4), *prp2 1; rrp6-Δ* (YCBA27) (lanes 7 and 8), *prp2 1; rat1 1* (YCBA57) (lanes 11 and 12), and *prp2 1; rat1 1; xrn1-Δ* (YCBA58) (lanes 13 and 14). Strains were grown in glucose medium at permissive temperature (23°C, odd numbered lanes) or for 3 hr at nonpermissive temperature (37°C, even numbered lanes). The positions of the degradation intermediates are indicated (A, A′, B, B*, and $A_{(An)}$). Each vertical set of panels corresponds to the same Northern blot hybridized with the 5 different probes. Probe numbers are on the left of the panels. To determine the relative levels of A, A′, and B by PhosphorImager quantification, the filter was hybridized successively with probe 1, to quantify A and A′ (from lane 2), probe 5 to quantify B (from lane 2), and probe PGK1 as a loading control. Values for A, A′, and B were expressed relative to band P to correct for probe efficiency and normalized to the corresponding PGK1 value to correct for loading.

revealed that species A extends to the 5′ end of the transcript, while B extends to the 5′ end of U24. The 5′ end of A′ was mapped at position +141 within the 5′ exon, most likely due to endonuclease cleavage.

In the *prp2 1* strain depleted of Rrp41p the A and A′ intermediates were lost, while the pre-mRNA (P) and the B intermediate were substantially accumulated (Figure 5B, lanes 2 and 4). The absence of Rrp p resulted in a similar, but somewhat weaker, phenotype (Figure 5B, lanes and 8). The residual A band appeared wider and was shifted up the gel (labeled $A_{(An)}$). Polyadenylated forms of the precursors to other snoRNAs and snRNAs are observed in strains lacking Rrp p (Allmang et al., 1999b; van Hoof et al., 2000), and $A_{(An)}$ probably corresponds to polyadenylated forms of species A. In both the Rrp41p and Rrp p depleted strains, band B was wider and shifted down the gel (labeled B* in Figure 5). Primer extension analysis showed that the 5′ end of B* was unaltered (data not shown). It is very likely that the accumulated B intermediate is slowly degraded from its 3′ end by a residual exonuclease activity, as has been observed for several other substrates in exosome mutants (Jacobs Anderson and Parker, 1998; Allmang et al., 1999b).

In the *prp2 1; rat1 1* strain at 37°C, levels of P, A and A′ were increased, as judged by Northern hybridization or primer extension (data not shown), consistent with the inhibition of 5′ degradation. However, the B form was still detectable, indicating residual 5′ processing activity (Figure 5B, compare lanes 10 and 12). Xrn1p is the only other known 5′→3′ exoribonuclease, and we therefore constructed a triple mutant strain carrying the *prp2 1* and *rat1 1* TS alleles together with an *URA3::xrn1* gene disruption (strain *prp2 1; rat1 1; xrn1-Δ*). In this strain the B intermediate was no longer detected by Northern hybridization, showing strong inhibition of the 5′ degradation pathway (Figure 5B, lane 14).

The processing of several other Rat1p substrates is more inhibited in strains also lacking Xrn1p. These include RNAs that are believed to be exclusively nuclear, the $27SA_3$ pre-rRNA, pre-rRNA spacer fragments and several pre-snoRNAs (Henry et al., 1994; Petfalski et al., 1998), indicating that there is a nuclear pool of Xrn1p, at least in *rat1 1* mutant strains.

Analysis of the degradation of *TEF4*, the host gene of the snoRNA snR38, also identified three decay intermediates, corresponding to P, A, and B (data not shown, but see Figure C). As for *BEL1*, the *TEF4* pre-mRNA is subject to both 5′ and 3′ degradation, with the 3′ pathway substantially more active. An A′ form was not present for *TEF4*, indicating that its degradation is entirely exonucleolytic.

The Northern data for the *prp2 1* strain at 37°C was quantified by PhosphorImager analysis (see legend to

Exhibit B

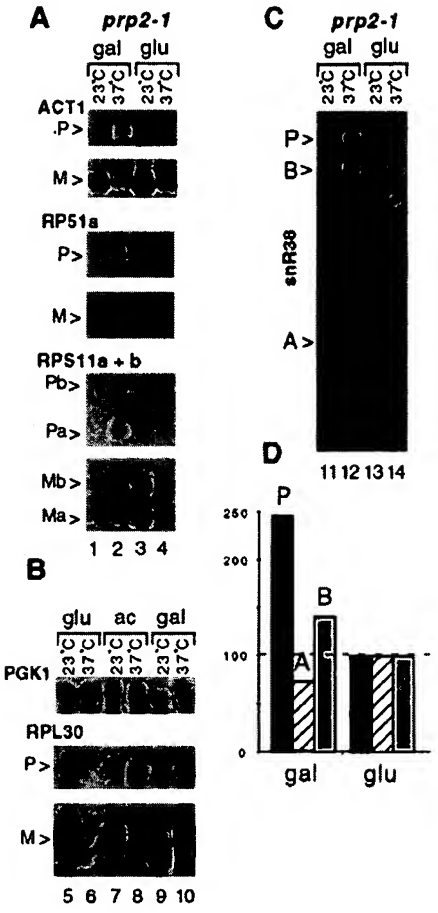

Figure . The 3′ Nuclear Pre-mRNA Decay Pathway Is Regulated by Carbon Source

Northern blot analysis of RNA extracted from strain *prp2 1* (YCBA20) grown in medium containing, as sole carbon source, galactose (gal), glucose (glu) or acetate (ac) following growth at permissive temperature (23°C) or 3 hr after transfer to 37°C. (A and B) Analysis of intron-containing pre-mRNAs. Probes used were as described in Figure 1. Position of the precursor (P) and mature form (M) are indicated. (C) Analysis of the *TEF4* pre-mRNA (the host gene for snR38) with a probe directed against the snR38 snoRNA (255). (D) PhosphorImager quantification of data from (C). Values for RNAs from strains grown in galactose based medium are expressed relative to the same intermediates observed upon growth in glucose medium, arbitrarily set at 100.

Figure 5). For *BEL1* the sum of A plus A′ was ~10-fold the level of B. For *TEF4* the A form was ~ .5 times more abundant than B. We conclude that the *BEL1* and *TEF4* pre-mRNAs are subject to both 5′ and 3′ degradation. In contrast to cytoplasmic mRNA turnover, 3′→5′ decay is the major nuclear pre-mRNA degradation pathway.

Nuclear Pre-mRNA Degradation Is a Regulated Process

In yeast, many pathways are subject to regulation dependent upon the available carbon source, and this is also the case for pre-mRNA turnover. Northern blot analysis of total RNA extracted from the *prp2 1* strain grown in galactose and glucose medium revealed a strong stabilization of all tested pre-mRNAs during growth in galactose medium (Figure A). Increased mRNA levels were seen for *RPS11a* and *RPS11b*, indicating that this is not a consequence of a further impairment in splicing on galactose medium. Transcription of ribosomal proteins is reduced on nutritional downshift due to the reduced growth rate (see Warner, 1989), and this pre-mRNA and mRNA accumulation is therefore probably being seen against a background of reduced synthesis. Growth on galactose medium did not inhibit other activities of the nuclear exosome; pre-rRNA processing and pre-rRNA spacer degradation (de la Cruz et al., 1998), processing of pre-snRNAs and pre-snoRNAs (data not shown).

Similar pre-mRNA stabilization was observed in the *prp2 1* strain grown in medium containing acetate (Figure B; lane 8 and data not shown) or maltose (data not shown) but not RS medium (Figure 1). Growth rates in galactose, maltose, and RS medium are similar, showing that this phenotype is not the consequence of slow growth. Notably, raffinose (a trisaccharide of galactose-glucose-fructose) and sucrose (a disaccharide of fructose and glucose) are metabolized by extra-cellular enzymes to release glucose, whereas galactose and maltose (a disaccharide of glucose) are converted to glucose by intracellular enzymes.

The decay intermediates of the *TEF4* pre-mRNA, the host gene for snR38, were also analyzed by Northern hybridization (Figure C); these data were quantified by PhosphorImager analysis (Figure D). During growth in galactose medium, the full-length precursor (P) was 2.5-fold more abundant than in glucose medium, while the 5′ processed species (B) was 1.4-fold more abundant. In contrast, the 3′ processed species (A) is 1.4-fold less abundant. The increased levels of P and B and decreased level of A reflect an inhibition of 3′→5′ degradation and increased 5′→3′ degradation in galactose based medium.

Discussion

A Nuclear Pre-mRNA urnover Pathway Competes with Splicing

Here we report the identification of a regulated pathway for the degradation of unspliced pre-mRNAs. This involves two mechanisms: 3′→5′ degradation by the exosome complex and 5′→3′ degradation by the exonuclease Rat1p. 3′→5′ degradation is the major pathway and was shown to require the activity of Rrp p, Rrp41p, Rrp44p, and Mtr3p, four components of the nuclear exosome complex, and presumably involves the entire complex.

There are clear similarities between the nuclear pre-mRNA degradation pathway and cytoplasmic mRNA turnover. Both pathways involve 3′→5′ degradation by the exosome, while 5′→3′ degradation is carried out by two homologous exonucleases: Rat1p in the nucleus and Xm1p in the cytoplasm (Hsu and Stevens, 1993; Muhlrad et al., , 1994, 1995; Jacobs Anderson and Parker, 1998). A clear difference is that cytoplasmic mRNA is predominately degraded 5′→3′ (Jacobs Anderson and Parker, 1998), whereas nuclear pre-mRNA is predominately degraded 3′→5′.

The in vitro activity of Rat1p is blocked by the presence of a cap structure (Stevens and Poole, 1995), suggesting that the 5′→3′ degradation pathway involves a

Table 1. Yeast Strains Used in This Study

Strain	Genotype	Reference/Note
D150	MATa *ura3-52 leu2-3,11, ade1-100 his4-519*	Mitchell et al., 1997
D271	MATα *ade2 his3 leu2 trp1 ura3*	Venema and Tollervey, 199
YDL401	MATa *his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108*	Lafontaine and Tollervey, 199
DJY3	MATa *ura3-52 prp2-1*	Plumpton et al., 1994
P118	MATa *his3Δ200 leu2Δ1 trp1 ura3-52 gal2 galΔ108 GAL10:: protA-RRP41*	Mitchell et al., 1997
YCA12	MATa *ade2-1 his3Δ200 leu2-3,112 trp1-1 ura3-1 can1-100 KI TRP1::rrp6*	Allmang et al., 1999a
D1 2	MATα *ura3-52 leu2Δ1 his3Δ200 rat1-1*	Amberg et al., 1992
D172	MATa *URA3::xrn1 rat1-1*	Henry et al., 1994
D342	MATa *ura3-52 mtr3-1*	Kadowaki et al., 1995
D348	MATα *ura3-52 lys2 rrp44-1*	P. Mitchell, A. Tartakoff, and D.T., submitted
YCBA20	MATa *ade his3 ura3 leu2 trp1 prp2-1*	This study: DJY3 xD271-21c
YCBA21	MATα *ade his3 ura3 leu2 trp1 prp2-1*	This study: DJY3 xD271-21d
YCBA27	as YCBA20 but *KI TRP1::rrp6*	This study
YCBA30	as YCBA20 but *GAL10::protA-RRP41*	This study
YCBA55	*his3 leu2 trp1 ura3 mtr3-1*	This study: D342xD271-5d
P213	MATa *leu2 rrp44-1*	P. Mitchell: D348xD150-3a
YCBA57	*ura3 leu2 his3 prp2-1 rat1-1*	This study: D1 2xYCBA21-2c
YCBA58	*prp2-1 rat1-1 URA3::xrn1*	This study: D172xYCBA21-2a
YCBA73	as D271 but *URA3::upf1*	This study
YCBA75	as YCBA20 but *URA3::upf1*	This study

nuclear decapping activity. Whether this is the cytoplasmic decapping enzyme Dcp1p (LaGrandeur and Parker, 1998) remains to be determined. It is notable that in many resident nuclear RNAs the cap structure is hypermethylated to trimethyl-guanosine. We speculate that one function of this modification is to resist nuclear decapping and subsequent degradation.

Levels of pre-mRNAs were generally low in strains in which splicing was blocked prior to the first catalytic step by the *prp2 1* mutation, indicating high turnover activity. All tested pre-mRNAs were strongly stabilized (up to 50-fold) by depletion of Rrp41p and clear but lower stabilization was seen in the absence of Rrp p or in strains carrying a TS-lethal mutation in Rat1p. In each case increased levels of the mature mRNAs were seen for some RNAs, showing competition between splicing and degradation of the pre-mRNA. This was confirmed using constructs in which the actin (*ACT1*) intron mutated at the 3′ splice site was fused to the *CUP1* reporter gene (Lesser and Guthrie, 1993). These mutations do not block spliceosome formation or the first catalytic step of splicing, forming the intron lariat-3′ exon and the free 5′ exon. TS mutations in the exosome components Rrp44p and Mtr3p or in Rat1p each substantially increased mRNA synthesis, indicating that the degradation of splicing intermediates is also in competition with splicing.

Competition between pre-mRNA degradation and splicing may be a normal feature of gene expression. Accumulation of all tested pre-mRNAs was seen in otherwise wild-type strains with reduced levels of Rrp41p. In the case of actin, a 5- to 8-fold increase in pre-mRNA levels was seen, suggesting that a significant fraction of the pre-mRNA is normally degraded. This was accompanied by a mild increase in mRNA levels compared to the nonspliced *PGK1* mRNA, strongly indicating that the increased pre-mRNA levels are not due to a splicing defect. Whether degradation is specifically activated by the inhibition of splicing remains to be determined. In the particular case of pre-mRNAs in which the intron-lariat branchpoint is incorrect, Prp1 p is implicated in determining whether the pre-mRNA continues along the splicing pathway or is targeted for degradation (Burgess and Guthrie, 1993).

Several lines of evidence show that pre-mRNA turnover takes place in the nucleus. Two components required for this pathway, Rat1p and Rrp p, are specifically localized in the nucleus (Johnson, 1997; Allmang et al., 1999a; Burkard and Butler, 2000). Moreover, mutations in Rrp41p/Ski p or Rrp p do not increase the stability of mRNAs in the cytoplasm (Benard et al., 1998; Jacobs Anderson and Parker, 1998; van Hoof et al., 2000) and this is presumably also the case for Rat1p. In contrast to the exonuclease mutants, inactivation of the cytoplasmic nonsense-mediated decay (NMD) pathway, that degrades cytoplasmic pre-mRNAs, did not stabilize pre-mRNAs in the splicing-deficient strain. Finally, for many pre-mRNAs, increased splicing was seen upon inhibition of turnover. It is very unlikely that these RNAs were localized in the cytoplasm, since splicing is a nuclear process.

Regulation of Pre-mRNA urnover

The availability of glucose, the normal and preferred carbon source for yeast, regulates a great number of metabolic activities via at least two signal transduction pathways (for recent reviews see Klein et al., 1998; Johnston, 1999). Analysis of unspliced pre-mRNAs and degradation intermediates in *prp2 1* strains showed that the activity of the 3′→5′ pre-mRNA degradation pathway depends upon the carbon source. Degradation activity was substantially higher in media containing glucose or raffinose + sucrose (which are converted to glucose by extracellular enzymes) than in galactose or maltose (which are converted to glucose by intercellular enzymes) or acetate. These observations suggest that the presence of extracellular glucose stimulates the degradative activity of the nuclear exosome on pre-mRNA substrates. This effect appears to be specific for pre-mRNA degradation; carbon source does not detectably alter "housekeeping" activities of the nuclear exosome in pre-rRNA processing, snRNA synthesis, snoRNA synthesis, or pre-rRNA spacer degradation.

It has frequently been observed that strains carrying TS-lethal mutations in splicing factors can grow at higher temperatures on glycerol or galactose media than

Exhibit B

on glucose medium (Tung et al., 1992; J. D. Beggs personal communication; B. Séraphin, personal communication), and this is also the case for the *prp2 1* strain that we use here (data not shown). We speculate that a reduced pre-mRNA degradation rate may contribute to this suppression by allowing increased time for splicing to occur.

We conclude that, like all other steps in gene expression from transcription to translation, the degradation of nuclear pre-mRNA is regulated. Alterations in nuclear RNA turnover in response to different physiological conditions may modulate mRNA synthesis rates. Most reported analyses of gene expression would not have distinguished between changes in transcription rate and alterations in nuclear RNA turnover.

Mammalian Pre-mRNA urnover and RNA Surveillance

It seems probable that the pathway described here will be conserved to mammals. Four human exosome components, hRrp4p, hRrp44p, hCsl4p (Mitchell et al., 1997; Baker et al., 1998; Shiomi et al., 1998; Allmang et al., 1999a), and hRrp41p (R. Brower, C. Allmang, E. Petfalski, D. Tollervey and W. van Venrooij, unpublished) can each function in yeast, as can mouse Rat1p (Shobuike et al., 1995). Indeed, this pathway is likely to be even more significant in humans, where regulated and alternative splicing events play important roles in gene expression. We predict that observed levels of mRNA synthesis will be significantly affected by the balance between splicing and degradation. Overall levels of nuclear pre-mRNA (i.e., hnRNA) degradation may also be much higher in mammals than in yeast. It has been estimated that only 2% of nuclear pre-mRNA is converted to cytoplasmic mRNA in mouse cells (Brandhorst and McConkey, 1974). Even allowing for intronic sequences, this suggests very active degradation.

There has been extensive analysis of mammalian RNA surveillance pathway that rapidly degrade pre-mRNAs that have undergone inaccurate or incomplete splicing (reviewed in Hentze and Kulozik, 1999; Hilleren and Parker, 1999; Mitchell and Tollervey, 2000). Many reports indicate that mammalian RNA surveillance is largely a nuclear activity (see Maquat, 1995; Hentze and Kulozik, 1999), and it seems very likely that the mechanism will involve homologs of the yeast components described here.

Experimental Procedures

Strains and Media

Rich, YP medium contained 2% peptone, 1% yeast extract and either 2% glucose, 2% galactose, 2% maltose, 2% sodium acetate or 2% raffinose + 2% sucrose (RS medium). Minimal YNB medium contained 0. 7% yeast nitrogen base (DIFCO) supplemented as required and 2% glucose.

Yeast strains used and constructed in this study are listed in Table 1. Strains YCBA20 (MATa; *prp2 1*) and YCBA21 (MATα; *prp2 1*) were obtained by sporulation of the diploid resulting from crossing DJY3 with D271. The *GAL10::ProtA-rrp41* allele in strains P118 and YCBA30 encodes a protein A-tagged fusion protein. To make strain YCBA30, the *HIS3-GAL10-ProtA-rrp41* cassette was PCR amplified from strain P118 with primers RRP41-1 (842) and RRP41-2 (843) and transformed into strain YCBA20. Correct integration was confirmed by PCR with primers RRP41-HIS3 (803) and RRP41-2. Expression of the ProtA-Rrp41p fusion was confirmed by Western blot and two transformants were selected (YCBA29 and YCBA30). To delete *RRP6*, a *Kluveromyces lactis TRP::rrp6-Δ* construct was PCR amplified from strain YCA12 with primers 5'RRP (834) and 3'RRP (835) and transformed into YCBA20. Correct integration was confirmed by PCR analysis with primers 3'RRP (835) and KI TRP (818) and two strains, YCBA27 and YCBA28, were selected for analysis. Strain YCBA57 was obtained by sporulation of the diploid resulting from crossing YCBA21 with D1 2. Strain YCBA58 was obtained by sporulation of the diploid resulting from crossing YCBA21 with D172. Strains YCBA55 and P213 were obtained by sporulation of diploids resulting from crossing D342 with D271 and D348 with D150, respectively. For the *upf1-Δ* strains, the *UPF1* ORF from −80 to +2090 was replaced by *S. cerevisiae URA3* in strains D271 and YCBA20. Correct integration was confirmed by PCR with primers UPF1-5' (8 4) and UPF1-3' (8 5) and restriction digestion of the resulting PCR product. In each case two strains were selected for analysis, YCBA73, YCBA74 and YCBA75, YCBA7 . Yeast transformation was performed as described (Gietz et al., 1995), except that % DMSO was added prior to heat shock and the final pellet was resuspended in 0.15 M NaCl.

Plasmids

Plasmids pJU83, pJU97, and pJU98 (Lesser and Guthrie, 1993) were kindly provided by C. Guthrie (UCSF, USA). Vector pJU83 carries an *ACT1-CUP1* construct with the wild-type 3' splice site (UAG), while pJU97 and pJU98 each have a single point mutation; UGG and UUG, respectively. Other mutants analyzed were at the 5' splice site; TTATGT (pSB30), CTATGT (pSB32), and GTAAGT (pSB33) and at the intron branchpoint: TACTACC (pSB38), TAATAAC (pSB47), TACAAAC (pSB49).

RNA Analysis

RNA extraction and Northern hybridization were performed as described (Beltrame and Tollervey, 1992). For Northern analysis, RNA was separated on 2% agarose-formaldehyde gels and transferred to Hybond N+. For strains *prp2 1; GAL::rrp41*, *prp2 1; rrp6-Δ*, *upf1-Δ* and *prp2 1; upf1-Δ*, Northern blot analyses were performed on RNA extracted from two independent transformants (YCBA29 and YCBA30, YCBA27 and YCBA28, YCBA73 and YCBA74, YCBA75 and YCBA7 , respectively). Primer extension was performed as described (Chanfreau et al., 1994) with minor modifications. 10 μg of total RNA was hybridized with 0.25 pmol of primer for 2 min at 100°C before being immediately frozen in liquid nitrogen. Reverse transcription was performed for 1 hr at 42°C. Sample loading was standardized to total RNA.

Oligonucleotides

The complete list of oligonucleotides used can be obtained from the authors (d.tollervey@ed.ac.uk) and is also available on the Cell web site as Supplementary Data (http://www.cell.com/cgi/content/full/102/ /7 5/DC1).

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Exhibit B

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Exhibit C

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The many pathways of RNA degradation.

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From the earliest comparisons of RNA production with steady-state levels, it has been clear that cells transcribe more RNA than they accumulate, implying the existence of active RNA degradation systems. In general, RNA is degraded at the end of its useful life, which is long for a ribosomal RNA but very short for excised introns or spacer fragments, and is closely regulated for most mRNA species. RNA molecules with defects in processing, folding, or assembly with proteins are identified and rapidly degraded by the surveillance machinery. Because RNA degradation is ubiquitous in all cells, it is clear that it must be carefully controlled to accurately recognize target RNAs. How this is achieved is perhaps the most pressing question in the field.

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